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13. ABSTRACT (Maximum 200 Words)

Clusterin is a multifunctional glycoprotein involved in tissue remodeling and apoptosis. However, recent studies have demonstrated that clusterin expression correlates with tumor grade in prostate cancer and in one retrospective study has been associated with tumor progression in breast cancer. Futhermore clusterin expression has been correlated with resistance to cytotoxic compounds such as TNF-α in prostate cancer, suggesting that clusterin may play a role in surviving cells.

In our studies, we have focused on determining whether clusterin plays a causative role in the progression of human breast cancer by promoting cell survival, increasing cell motility and resistance to cytotoxic drugs.

Our studies have utilized an ER-α positive non-invasive MCF-7 cell line, an MCF-7 cell line genetically engineered to overexpress clusterin (MCF-7CLU) and an ER-alpha negative breast negative invasive SUM-159PT cell line. Our major finding to date are that MCF-7CLU cells express 5-10 fold higher levels of intra- and extra-cellular clusterin as compared to parental MCF-7 cells. The MCF-7CLU cell line exhibits increased growth rates, altered cellular morphology, a dramatic increase (10-fold) in invasive potential and a delayed apoptotic response to cytotoxic compounds such as TNF-α and Tamoxifen in comparison to the parental MCF-7 cells.

In vivo nude mouse studies have determined that MCF-7 CLU tumors exhibit higher growth kinetics, and decreased sensitivity to Tamoxifen as compared to parental MCF-7 tumors. Furthermore observations by gross morphology have demonstrated metastatic spread of MCF-7CLU cells to the lungs of nude mice, a phenomenon never observed in the parental MCF-7 xenografts.
Our data clearly demonstrate a role for clusterin in breast tumor promotion and resistance to possible therapeutic compounds.

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INTRODUCTION

The development of a metastatic, hormone independent, and drug resistant phenotype is responsible for a hight percentage of treatment failures among breast cancer patients. Therefore understanding the molecular mechanisms of metastasis is crucial for the design and effective use of novel therapeutic strategies to combat cancer.

Clusterin is a highly conserved 70-80 kDa heterodimeric, disulphide linked sulphated glycoprotein. The multifunctional nature of this protein is reflected by its expression pattern. Increases in clusterin mRNA and protein levels have been consistently detected during apoptosis in the kidney, prostate, breast, and retinal tissue both *in vitro* and *in vivo* [1-3]. On the basis of its elevated expression in apoptotic tissues, it was originally proposed that the protein might be causally involved in apoptosis. However there is accumulating evidence that the expression of clusterin is restricted to surviving cells. It has been demonstrated that intracellular levels of clusterin correlated with tumour grade in prostate cancer [4].

In breast cancer cells where clusterin expression or protection has been less extensively studied, only one study has indicated that clusterin expression is associated with large tumour size, and with the progression from primary carcinoma to metastatic carcinoma in lymph nodes [5]. The increased clusterin expression in breast carcinomas correlates inversely with the apoptotic index, which suggests that clusterin gene expression is not a prerequisite to cellular death by apoptosis but may have a role in tumorigenesis and progression of human breast carcinoma. However, there is little other data available on the effects of clusterin expression and secretion on human breast cancer growth, invasion and sensitivity to cytotoxic drugs *in vitro* or *in vivo*.

The identification of a functional conserved heat shock element in the clusterin promoter and the demonstration that clusterin is a heat shock inducible gene is consistent with the idea that clusterin is involved in coping with the consequences of stresses related to tissue injury [6],[5, 7, 8]. Sensitivity to TNF-alpha in PC3 and LNCaP prostatic cancer cells has been shown to be regulated by extracellular levels of clusterin [9]. It has also been shown that overexpression of the clusterin gene in human androgen-dependent LNCaP prostate cancer cells by stable transfection rendered them highly resistant to androgen ablation *in vivo* and decreased radiosensitivity. Furthermore, *in vivo* administration of anti-sense oligonucleotides and either paclitaxel or mitoxantrone significantly decreased the primary tumour volume by approximately 70% respectively compared to mismatch control oligonucleotides plus either paclitaxel or mitoxantrone [10-16]. Similar results have been observed in human renal cell cancer using antisense oligonucleotides targeting clusterin [17].

These findings demonstrate that clusterin overexpression confers resistance to cytotoxic chemotherapy and that inactivation of clusterin using anti-sense technology might offer a novel strategy to improve results of radiation therapy for prostate cancer patients. The aim of this research proposed here is to determine whether clusterin expression in breast cancer cells confers a similar survival advantage, helping to accelerate the progression to a more invasive drug-resistant phenotype.

ANNUAL SUMMARY-BODY

Based on our literature searches and preliminary data carried out for the grant proposal we suggest that clusterin expression may play a role in tumorigenesis and progression of human breast carcinomas. The aim of this research proposed here is to determine whether clusterin expression in breast cancer cells confers a survival advantage and helps to accelerate the progression to a more, invasive drug-resistant phenotype.

Although five-year survival rates for locally and disseminated breast cancer are improving, the survival rate for women with distant metastases is only 20%. This appears to be due to the acquisition of a more aggressive phenotype, characterized by invasive/metastatic foci, which are resistant to endocrine manipulation and chemotherapeutic intervention. Previously in our grant proposal we were utilizing two breast cancer cell lines from different stages of breast cancer progression and with different sensitivities to cytotoxic drugs to examine the role of clusterin in the progression of breast cancer to a metastatic endpoint. MCF-7 cells are ER-positive, non-invasive breast cancer cell line that form localized tumors after orthotopic injection into the mammary fat pads of nude mice in the presence of oestradiol. SUM-159PT cells on the other hand are ER-negative cells and form invasive tumors in ovariectomized nude mice even in the absence of oestradiol [18]. MCF-7 and SUM-159PT cells exhibit varying sensitivities to cytotoxic compounds, for example the ER-negative SUM-159PT cells are resistant to the cytotoxic drug TNF-α whereas the ER-positive MCF-7 cells are highly sensitive.

My research over the previous two years has expanded our study on the role of clusterin in breast cancer by creating an MCF-7 cell line genetically engineered to overexpress clusterin (MCF-7CLU) to directly compare the effects of clusterin overexpression on the MCF-7 cell line, whilst also using the SUM-159PT cells as a control model for invasive, metastatic and cytotoxic drug resistant breast cancer.

Characterization of the MCF-7CLU cell line displayed several important factors. Firstly we examined the basic morphology of these cells using phase microscopy. As demonstrated in Fig. 1, MCF-7CLU cells exhibit more cell to cell contacts and form a tighter knit monolayer (Panels D, E and F) as compared to the islands of cells exhibited by the parental MCF-7 cells (Panels A, B and C).

Second, the level of extracellular clusterin expression is approximately 15-fold higher in the MCF-7CLU cells that the parental MCF-7 cells and is approximately the same as that seen in the SUM-159PT cells (Fig. 2). Treatment of MCF-7 cells with TNF-α induces an 8-fold increase in extracellular clusterin expression as compared to ethanol treated controls. No increase in clusterin expression is observed in TNF-α treated SUM-159PT cells indicating constitutive high level expression of clusterin in these cells (Fig. 2).

Similarly, the level of intracellular clusterin expression in MCF-7CLU cells is approximately 10-fold higher than that observed in parental MCF-7 cells. Treatment of MCF-7 cells with varying concentrations of TNF-α induced a modest increase in clusterin expression levels (Fig 3A). In MCF-7CLU cells, the levels of clusterin remain constitutively high upon treatment with varying concentrations of TNF-α. Treatment of either MCF-7 and MCF-7CLU cells with either 1 or 2.5 μM Tamoxifen induced a marked increase in clusterin expression in both cell lines although overall levels of clusterin in MCF-7CLU cells remain at least 10-fold higher than in parental MCF-7 cells (Fig 3B).

Third, analyses of proliferation rates (crystal violet analyses) demonstrated that MCF-7CLU cells are substantially resistant to TNF-α when compared to parental MCF-7cells (Fig. 4A). Furthermore MCF-

7CLU cells are relatively resistant to low levels (1 μM Tamoxifen) of the selective estrogen receptor modulator (SERM) tamoxifen, even though these cells retain the estrogen receptor (ER) (Fig 4B). Examination of the expression of ER-α levels in both MCF-7 and MCF-7CLU cells demonstrate similar basal levels of expression. Treatment of MCF-7 cells with either 1 or 2.5 μM Tamoxifen resulted in a marked downregulation of ER-α relative to control treated MCF-7 cells. In contrast little or no downregulation of ER-α was observed in Tamoxifen treated MCF-7CLU cells, indicating a delayed onset of growth arrest and apoptosis (Fig. 5)

Expanding on the above data relating to resistance of MCF-7 CLU cells to the effects of TNF-α and Tamoxifen, cell cycle analyses was carried out on both MCF-7 and MCF-7CLU cells treated with either 5 ng/ml TNF-α or 5 μM Tamoxifen. Cells were harvested, fixed and stained with propidium iodide. As demonstrated in Table 1, MCF-7 CLU control cells exhibit a greater percentage of cells in S-phase (35%) as compared to MCF-7 parental cells (27%). Treatment with 5 ng/ml TNF-α or 5 μM Tamoxifen caused a marked increase in the population of MCF-7 cells in the G1 phase of the cell cycle (73% and 78% respectively) as compared to control treated cells (60%). In contrast there was little effect on cell cycle kinetics of MCF-7 CLU cells treated with either 5 ng/ml TNF-α or 5 μM Tamoxifen (Table1).

Analyses of the expression of the cell cycle regulatory protein p21 demonstrated a marked increase in p21 levels in TNF-α treated MCF-7 cells relative to EtOH treated control cells. In contrast, no difference in p21 expression levels between control and TNF-α treated MCF-7CLU cells was observed (Fig. 6). This data correlates positively with the above cell cycle data.
We are currently examining the expression levels of p21 in Tamoxifen treated MCF-7 and MCF-7CLU cells.

Furthermore, analyses of apoptotic related proteins revealed that there was a marked delay in the onset of apoptosis in MCF-7CLU cells upon treatment with TNF-α and Tamoxifen. Treatment of MCF-7 cells with 5 ng/ml TNF-α resulted in a translocation of cytosolic Bax to the mitochondrial membrane and subsequent cleavage to t-Bax. In contrast no difference in Bax localization was observed in TNF-α treated MCF-7CLU cells relative to control treated cells (Fig 7).

Our data clearly demonstrate that high levels of clusterin expression confers resistance or a delayed response to the TNF-α and Tamoxifen. These results are consistent with data from prostate cancer models systems that have shown that overexpression of the clusterin gene in human androgen-dependent LNCaP cancer cells rendered them highly resistant to androgen ablation *in vivo* [13,14].

Our next aim was to examine if the increased expression of clusterin in MCF-7CLU cells confers a survival advantage and helps accelerate the progression to a more invasive metastatic phenotype. As demonstrated in Fig. 8, in a modified Boyden chamber invasion assay, MCF-7 cells invade minimally, in contrast to the highly invasive SUM-159PT cells. Overexpression of clusterin in MCF-7 cells caused a 10-fold increase in the level of invasion as compared to parental MCF-7cells.

Based on this *in vitro* data, we assessed the role of clusterin in tumor formation, doubling times, metastatic potential and sensitivity to the SERM tamoxifen after orthotopic injection of the MCF-7CLU cells into the mammary fat pad of oestrogen supplemented ovariectomized nude mice as previously described in the study design laid out in last years annual report (Fig 9).

As demonstrated in Fig.10, MCF-7CLU tumor growth kinetics are significantly higher than those observed in the parental MCF-7 xenografts. Furthermore histological analyses of tissues sections from various potential metastatic sites revealed metastatic spread of MCF-7CLU cells to the lymph

nodes (60% of mice) and to the lungs (50% of mice) of nude mice, a phenomenon never observed in the parental MCF-7 xenografts. A representative H&E of metastases to the lung of nude mice orthotopically inoculated with MCF-7CLU cells is shown in Fig 11.

Treatment with Tamoxifen (provided as a slow release pellet) for 5 weeks causes a highly significant 63% reduction in MCF-7 tumor volume as compared to parental MCF-7 xenografts, while MCF-7CLU xenografts show a 30% reduction which was not statistically significant compated to placebo treated MCF-7CLU xenografts (Fig. 12).

Histological analysis of MCF-7 tumors demonstrate a decrease in the number of epithelial cells in Tamoxifen treated tumors. This decrease in the epithelial component is associated with increased DNA fragmentation as assessed by TUNEL staining. In contrast Tamoxifen treated MCF-7CLU tumors exhibit a similar morphology to that observed placebo treated control tumors (Data not shown). Currently we are quantitating the relative levels of DNA fragmentation in placebo and Tamoxifen treated MCF-7 and MCF-7CLU tumors.

These data demonstrate that overexpression of clusterin increases the rate of primary tumor growth, metastatic progression and resistance to currently used therapeutic agents.
Taken together these data are the first to clearly demonstate in breast cancer cells that clusterin expression confers a survival advantage and helps to accelerate the progression to a more invasive drug-resistant phenotype. Our data are consistent with the single report in breast tumor specimens that clusterin expression is associated with large tumor size, lack of estrogen and progesterone receptor status and with the progression from primary carcinoma to metastatic carcinoma in lymph nodes [5].

In addition since clusterin is a secreted protein and required for cell survival, support for this hypothesis would suggest that clusterin is an excellent target for immumotherapy.

STATEMENT OF WORK

Task 1: To quantitate the relative levels of clusterin secretion in conditioned media of either untreated (control) or treated (TNF-α, Vitamin D) SUM-159PT and MCF-7 cells.

My studies so far have allowed me to measure using western blot analysis the relative secretion of clusterin in conditioned media and lysates of control, TNF-α and Tamoxifen treated MCF-7 and the MCF-7CLU cells. We wanted to examine the relative levels of clusterin in Tamoxifen treated cells as Tamoxifen is the most commonly used clinical endocrine therapy for women with breast cancer and so to examine the role of clusterin in relation to sensitivity/resistance to tamoxifen is therefore of great clinical importance.

Our future studies plan to repeat the above studies vitamin D treated cells.

Task 2: To determine the effects of AS clusterin on growth, invasion and sensitivity to cytotoxic compounds in SUM-159PT and MCF-7 cells.
Although we have not examined directly examined the effects of AS clusterin on growth, invasion and sensitivity to cytotoxic compounds in SUM-159PT and MCF-7 cells we have however created an MCF-7 cell line that has been engineered to overexpress clusterin and our results have demonstrated that overexpression of clusterin leads to increased survival, invasion and resistance to cytotoxic compounds (as measured by flow cytometry and analyses of apoptosis-related proteins). Future studies this year would include transfection of SUM-159PTGFP cells with AS clusterin under the

control of the Tet/on system to determine if inhibiting clusterin expression in invasive and cytotoxic resistant cells can revert these phenotypes.

Task 3: To determine the effects of AS clusterin on growth, invasion and sensitivity to cytotoxic comounds in SUM-159PT and MCF-7 tumors.

Although we have not examined directly examined the effects of AS clusterin on growth, invasion and sensitivity to cytotoxic compounds in SUM-159PT and MCF-7 tumors, we have assessed the role of clusterin in tumor formation, doubling times, metastatic potential and sensitivity to the SERM tamoxifen after orthotopic injection of the MCF-7CLU cells into the mammary fat pad of oestrogen supplemented ovariectomized nude mice as compared to parental MCF-7 tumors.

We intend this year to complete the above task utilizing SUM-159PTGFP AS clusterin cells under the control of the Tet/on system to determine if inhibiting clusterin expression affects the growth, metastatic spread and sensitivity of SUM-159PT^{GFP} tumors to cytotoxic compounds.

Task 4: Manuscript Preparation

Currently we are in the process of writing up a manuscript based on the MCF-7CLU results described above.

KEY RESEARCH ACCOMPLISHMENTS

- Creation by our laboratory of an MCF-7 cell line genetically engineered to overexpress clusterin, designated MCF-7CLU.

- Characterization of MCF-7CLU cells reveals several important factors. MCF-7CLU cells express 5-10 fold higher levels of intra- and extra-cellular clusterin as compared to parental MCF-7 cells.

- The MCF-7CLU cell line exhibits increased growth rates, altered cellular morphology and delayed sensitivity to cytotoxic compounds such as TNF-α and the selective estrogen receptor modulator (SERM) tamoxifen when compared to parental MCF-7 cells.

- *In vitro* invasion assays demonstrate a dramatic increase (10 fold) in the invasive potential of the MCF-7CLU cells over the non-invasive MCF-7 cells.

- MCF-7CLU tumor growth kinetics are significantly higher than those observed in the parental MCF-7 xenografts.

- Metastatic spread of MCF-7 CLU cells to the lymph nodes and lungs of nude mice was observed, a phenomenon never observed in parental MCF-7 xenografts.

- Treatment with Tamoxifen (provided as a sustained release pellet) for 5 weeks causes a significant 63% reduction in MCF-7 tumor volume as compared to placebo treated MCF-7 xenografts, while MCF-7CLU xenografts show a 30% reduction which is not statistically significant compared to placebo treated MCF-7CLU xenografts.

- Taken together our data are the first to clearly demonstrate that clusterin expression confers a survival advantage and helps to accelerate the progression to a more invasive drug-resistant phenotype in breast cancer cells.

REPORTABLE OUTCOMES

- **Development of a new cell line:** In our laboratory we have a well designed protocol for overxpression of genes using Gateway technology. Using Invitrogen's Gateway Technology, which allows for the transfer of DNA among different vectors based on lambda-phage recombination, the clusterin gene was inserted into a donor vector (pDONOR 201) and then subcloned into a mammalian expression vector which was then stably transfected into the MCF-7 cell line using Lipofectamine 2000 transfection reagent. This new cell line is designated **MCF-7CLU**.

- **Poster and invited oral presentation of this work at the European Association for Cancer Research (EACR) meeting, Innsbruck, Austria, July 3-6, 2004.**
  Authors: Flanagan L, Whyte L, Dawson J, Ryan A, Lopez J* and Tenniswood M.
  Title: Clusterin overexpression confers resistance to cytotoxic drugs and triggers metastatic progression in breast cancer cells.

- **Invited oral presentation of this work at the Annual Research Symposium –Common Molecular Mechanisms in Inflammation and Malignancy at the Education and Research Centre, St. Vincent's University Hospital, Dublin 4, Ireland; Nov 12, 2004.**
  Title: Modelling Breast Cancer Progression and Treatment *in vivo.*

CONCLUSIONS

The overall aim of our studies was to investigate whether clusterin expression plays a causative role in the progression of human breast carcinoma.

Our data are the first to show in breast cancer cells and tumors that high extracellular clusterin expression plays a role in tumorigenesis and progression of human breast carcinomas by promoting cell survival, increasing cell motility and invasion and resistance to cytotoxic drugs.

Our data therefore would illustrate the potential utility of combined treatments of anti-sense (AS) clusterin (or antibodies directed against clusterin) and other therapeutic agents for patients with either hormone-dependent or hormone-refractory breast cancer. For patients with hormone-dependent cancer, we would predict lower levels of therapeutic agents in AS clusterin would yield lower a similar result as higher levels of drug alone, thereby minimizing some of the potentially unfavorable side effects of many currently used therapeutic agents. In the case of hormone-refractory cancers we would predict enhanced sensitivity to cytotoxic drugs and an inhibition of metastatic spread of breast cancer cells. In addition since clusterin is a secreted protein and required for cell survival, support for this hypothesis would suggest that clusterin is an excellent target for immunotherapy.

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APPENDIX - FIGURES

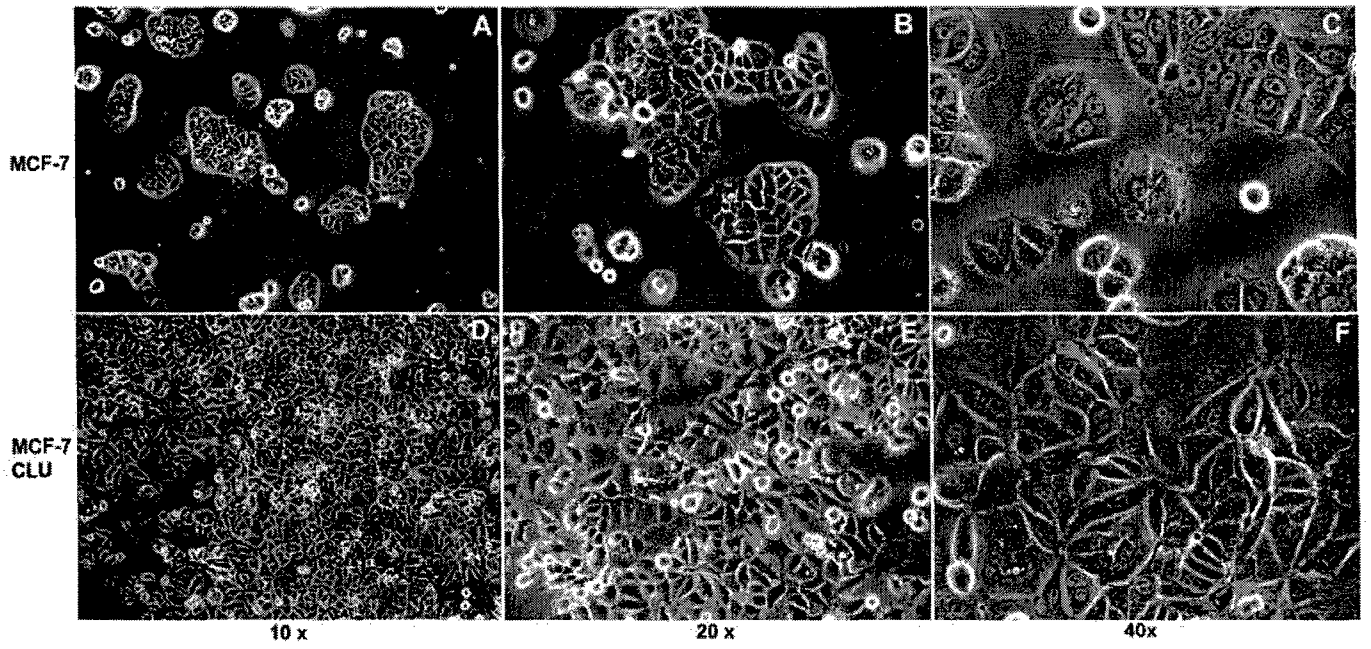

Figure 1: Phase Microscopy of MCF-7 and MCF-7CLU cells. MCF-7 and MCF-7 cells were plated on Lab-Tek cell culture inserts at 20,000 cells/ml and allowed to grow for 72 h. Cells were fixed with 4% formalin and photographed using phase microscopy at 10 x, 20 x and 40 x magnification.

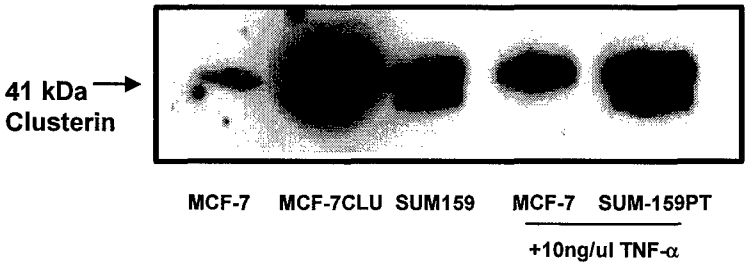

Figure 2: Western Blot Analysis of Clusterin in conditioned Media. MCF-7, MCF-7CLU and SUM-159PT cells were ethanol control treated or MCF-7 and SUM-159PT were treated with 10 ng/ml TNF-α for 24 h and separated on a 12.5% SDS-PAGE gel and immunoblotted with a mouse monoclonal antibody directed against clusterin.

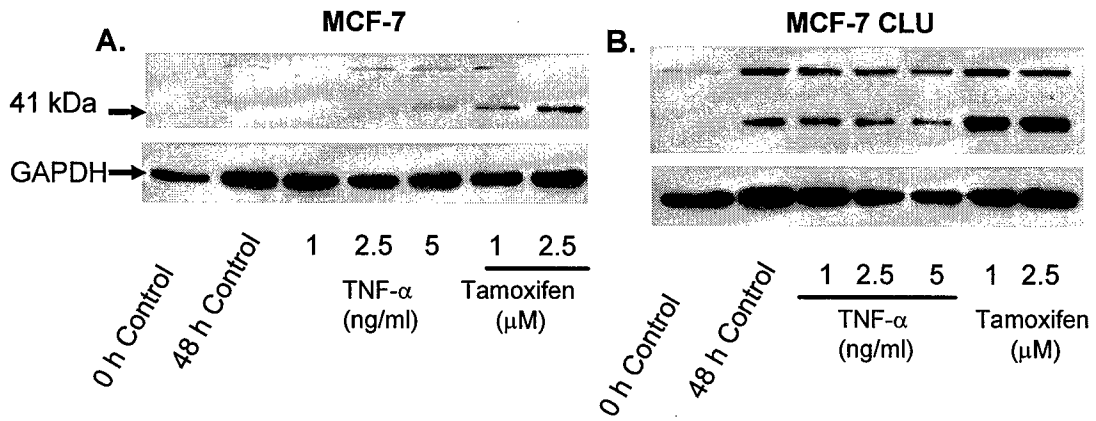

Figure 3: Western Blot Analysis of Clusterin in Cell Lysates: MCF-7 and MCF-7CLU cells were treated with either ethanol (control), 5 ng/ml TNF-α or 5 μM Tamoxifen for 24 h. Cell lysates were extracted and separated on a 12.5% SDS-PAGE gel and immunoblotted with a mouse monoclonal antibody directed against clusterin (RDI-CLUSTabm-41D- Research Diagnostic Labs) and GAPDH.

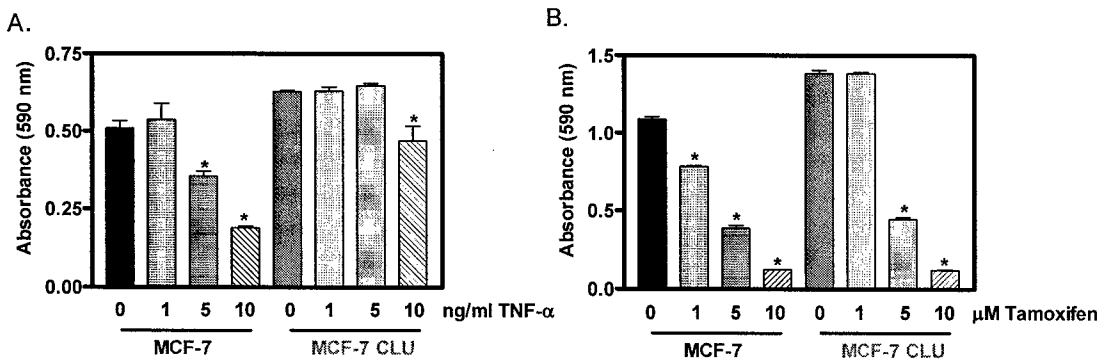

Figure 4: Effects of TNF-α and Tamoxifen on MCF-7 and MCF-7CLU cell growth. Cells were plated at 20,000 cells/ml and two days after plating cells were treated with either ethanol vehicle or 1, 5 or 10 ng/μl TNF-α for 48 h or 1, 5 and 10 μM Tamoxifen for 72 h. Total cell number was determined by crystal violet staining. Data represent mean ±SEM of four values per time point. *, $P<0.01$; ethanol control vs TNF-α/Tamoxifen treated.

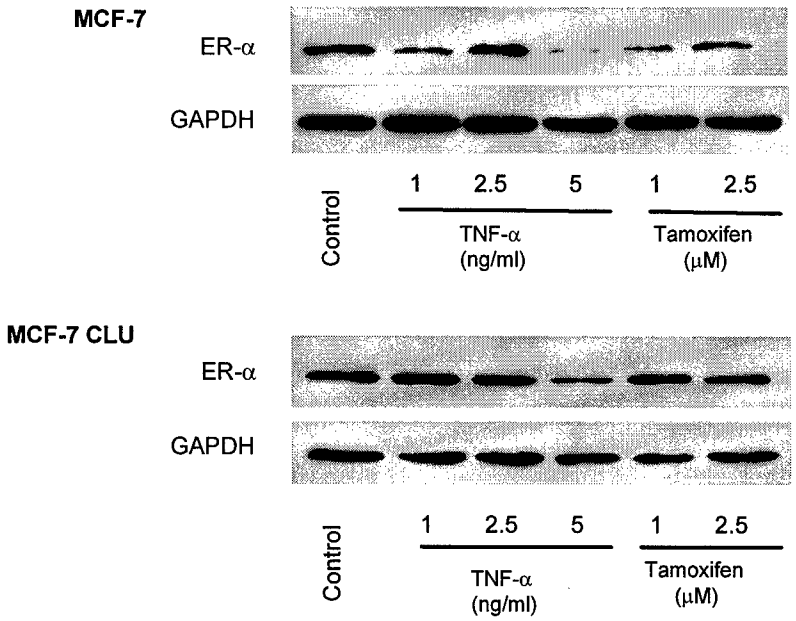

Figure 5: Western blot analysis of ER-α in MCF-7 and MCF-7 cells treated with TNF-α or Tamoxifen. MCF-7 and MCF-7CLU cells were treated with either ethanol (control), 1, 2.5 or 5 ng/ml TNF-α or 1, and 2.5 μM Tamoxifen for 24 h. Cell lysates were extracted and separated on a 10 % SDS-PAGE gel and immunoblotted with a mouse monoclonal antibody directed against ER-α (6F-11-Vector Labs) and GAPDH.

Percentage of Cells	G_0/G_1		S		G_2/M	
	MCF-7	MCF-7CLU	MCF-7	MCF-7CLU	MCF-7	MCF-7CLU
Control	60.6	54.1	27	34.2	12.4	11.7
Tamoxifen	78.2	60	12.3	31.7	9.5	8.3
TNF-α	72.8	56.3	15.3	41.9	11.9	1.8

Table 1: Cell cycle analyses of MCF-7 and MCF-7CLU cells treated with TNF-α and Tamoxifen. MCF-7 and MCF-7CLU cells were treated with either 5 ng/ml TNF-α or 5 μM Tamoxifen for 24h. Cells were harvested by trypsinization, fixed in ethanol and stained with propidium iodide and analyzed by flow cytometry.

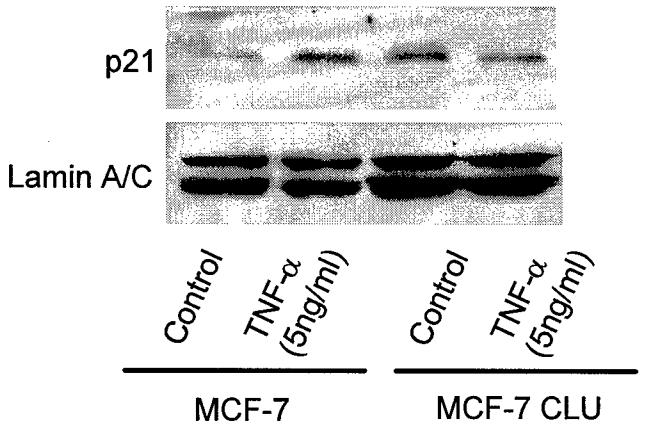

Figure 6: Western Blot analysis of p21 expression in TNF-α treated MCF-7 and MCF-7CLU cells. MCF-7 and MCF-7 CLU cells were treated with either ethanol control or 5ng/ml TNF-α for 24h. Nuclear extracts were extracted and separated on a 15 % SDS-PAGE gel and immunoblotted with mouse monoclonal antibodies directed again p21 (Santa Cruz) and lamin a/c (Santa Cruz) (loading control).

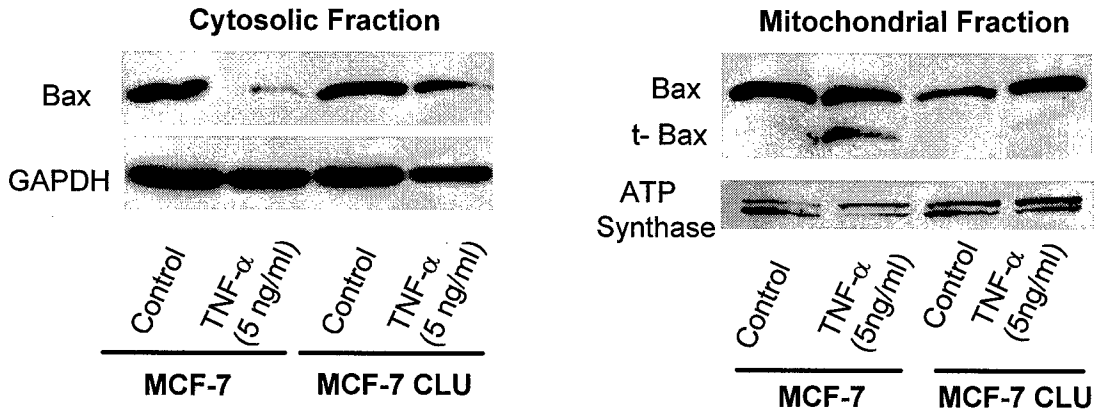

Figure 7: Effect of Clusterin Overexpression on Bax Translocation and Cleavage. Cytosolic and mitochondrial fractions were prepared from parental MCF-7 and MCF-7CLU cells after 48 h treatment with either ethanol control or 5 ng/ml TNF-α. Proteins fractions were separated on 15 % SDS-PAGE gels and were immunoblotted with antibodies directed against Bax (Calbiochem). Protein samples were normalized by comparison to GAPDH (cytosolic fraction) and ATP-synthase (mitochondrial fraction).

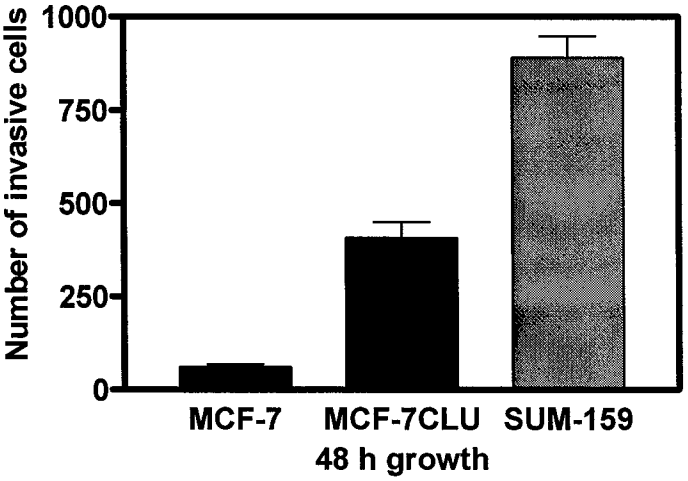

Figure 8: Invasive Potential of MCF-7, MCF-7CLU and SUM-159PT cells. Cells were analyzed in the Boyden chamber invasion assay. 50,000 cells/ml were plated in each insert and cells were allowed to grow for 48 h. Cells on the underside of the insert were fixed with 1% gluteraldehyde, stained using crystal violet, allowed to dry and cells counted using a light microscope. Data represent mean ±SEM of four values per time point.

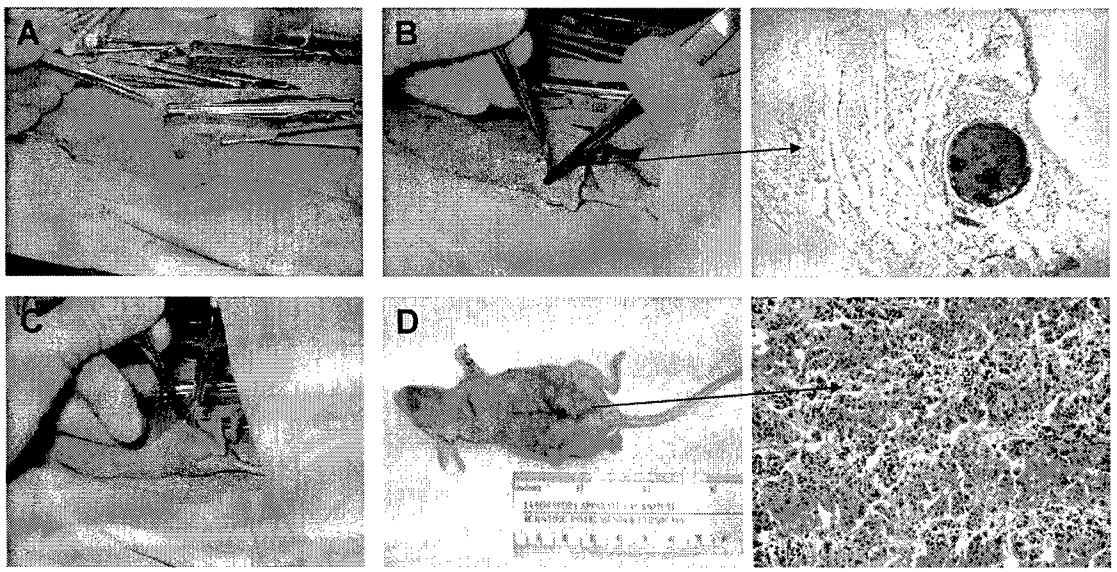

Figure 9: Inoculation of breast cancer cells into the inguinal mammary fat pad of nude mice. MCF-7 and MCF-7CLU cells suspended in Matrigel were injected into mammary fat pad using a 26 gauge needle and a 1 cc syringe.

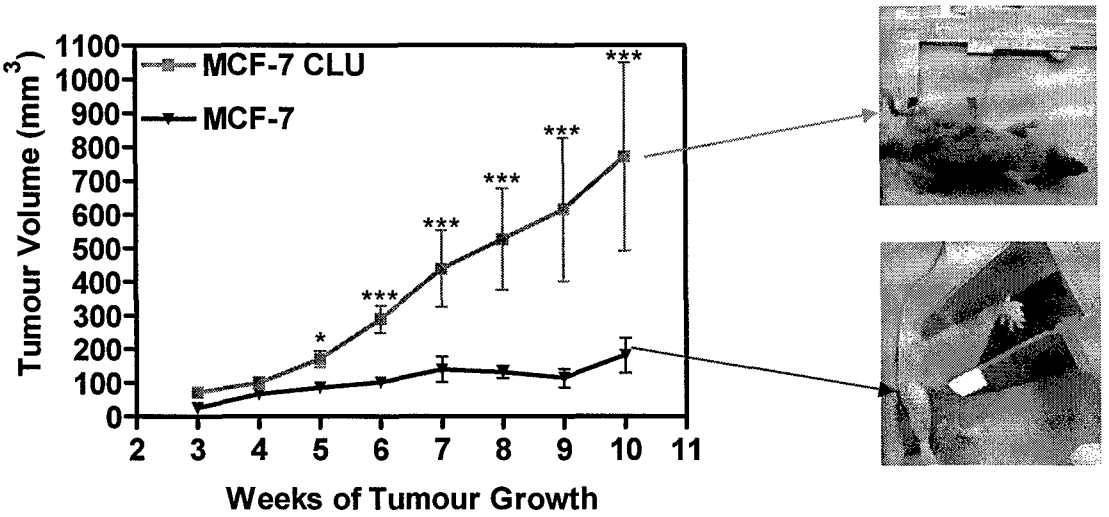

Figure 10: Effect of clusterin overexpression on tumor growth. MCF-7 and MCF-7CLU tumor volumes were calculated weekly by calipers measurements. Tumor volumes * P <0.05; ***P<0.001; MCF-7CLU vs MCF-7 tumor volume.

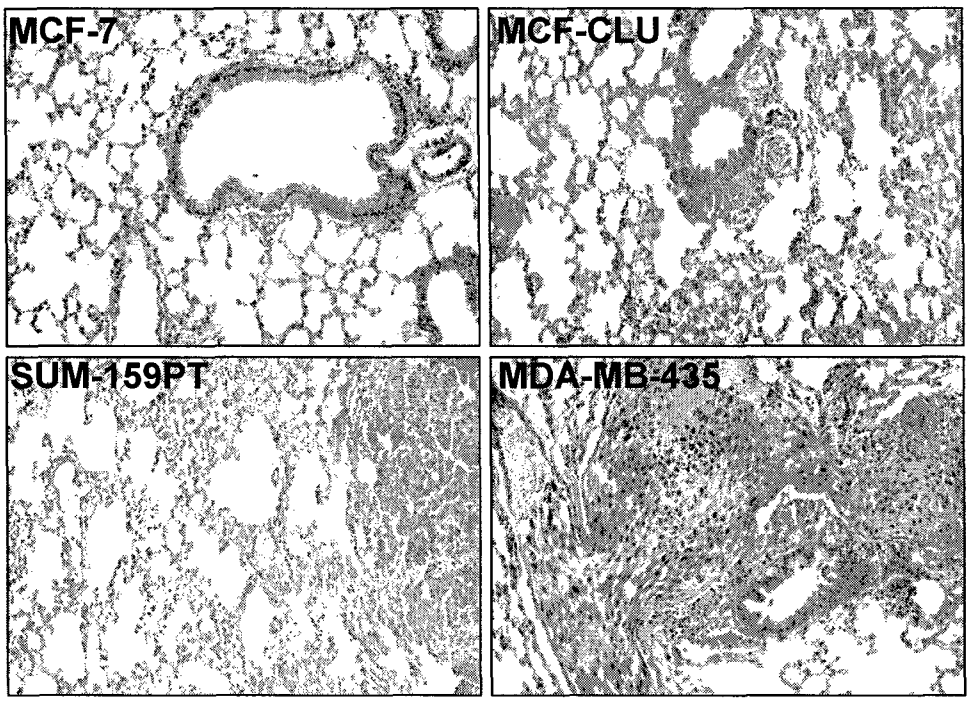

Figure 11: Hematoxylin and Eosin staining of lung tissue from nude mice inoculated orthotopically with MCF-7 and MCF-7CLU breast cancer cells. 5 μM paraffin embedded tissues sections were routinely stained using H&E staining. Lung sections from mice inoculated with the metastatic SUM-159PT and MDA-MB-435 cells were used as a positive control. Photographed at 10 x magnification.

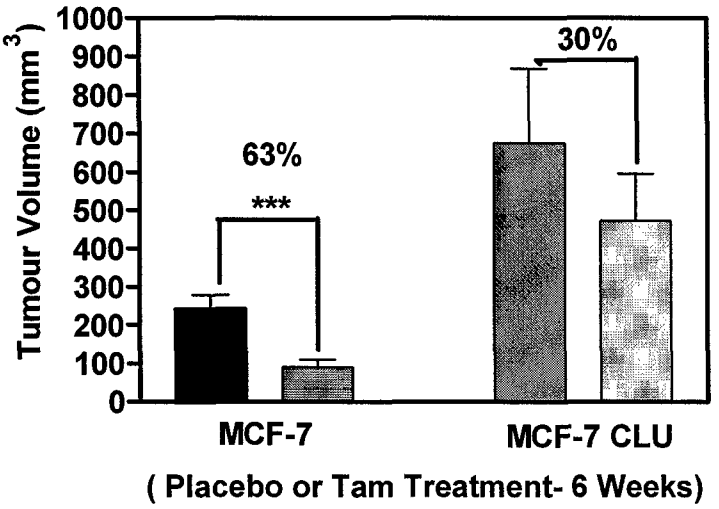

Figure 12: Effect of clusterin overexpression on sensitivity of MCF-7 tumors to Tamoxifen. MCF-7 and MCF-7CLU tumor volumes from placebo control or Tamoxifen (15mg/90day slow release pellet) treated mice were calculated weekly by caliper measurements. *** $P<0.001$; placebo vs Tamoxifen treatment.